

FINAL REPORT

Test Facility Study No. 511885

**Activated Sludge Respiration Inhibition Test
(Carbon and Ammonium Oxidation)
with
MLA-3202**

SPONSOR:

Chemtura Corporation
199 Benson Road
MIDDLEBURY, CT 06749
USA

TEST FACILITY:

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The Netherlands

06 December 2016

Page 1 of 24

TABLE OF CONTENTS

LIST OF TABLES	2
LIST OF FIGURES	3
LIST OF APPENDICES	3
1. STATEMENT OF GLP COMPLIANCE.....	4
2. TEST FACILITY QUALITY ASSURANCE STATEMENT	5
3. SUMMARY	6
4. INTRODUCTION	7
4.1. Study schedule	7
4.2. Purpose.....	7
4.3. Guidelines	7
4.4. Retention of records and materials	7
4.5. Responsible personnel	7
4.5.1. Test facility	7
4.5.2. Sponsor Representative.....	7
4.6. Definitions.....	8
5. MATERIALS AND METHODS	8
5.1. Test item.....	8
5.1.1. Test item information.....	8
5.1.2. Study specific test item information	8
5.2. Vehicle information	8
5.3. Reference item	9
5.3.1. Reference item information	9
5.3.2. Reference item – preparation of stock and test concentrations.....	9
5.4. Test item – preparation of test solutions	9
5.5. Combined limit/range-finding test.....	9
5.6. Test system.....	10
5.7. Test procedure and conditions	10
5.8. Interpretation.....	12
5.8.1. Calculations.....	12
5.8.2. Acceptability of the test	13
5.9. List of deviations.....	13
5.9.1. List of study plan deviations	13
5.9.2. List of standard operating procedures deviations	13
6. ELECTRONIC SYSTEMS FOR DATA ACQUISITION.....	14
7. RESULTS	15
7.1. Inhibition of the respiration rate	15
7.2. Determination of effect parameters	15
7.3. Experimental conditions	15
8. CONCLUSION	15

LIST OF TABLES

Table 1	Experimental set-up	10
Table 2	Effect parameters	15

Table 3	Results: respiration rate/inhibition, pH values.....	16
Table 4	Estimated parameters of the 3-param. normal CDF	22
Table 5	Analysis of Variance for the 3-param. normal CDF.....	22
Table 6	EC-values determined for 3,5-dichlorophenol.....	22

LIST OF FIGURES

Figure 1	Recording of oxygen consumption (Blank/Nitrification controls, Reference item).....	18
Figure 2	Recording of oxygen consumption (MLA-3202)	19
Figure 3	Recording of oxygen consumption (MLA-3202 with nitrification inhibitor)	20
Figure 4	Recording of oxygen consumption (Blank / Nitrification controls)	21
Figure 5	Concentration-effect curve showing the influence of 3,5-dichlorophenol on total respiration rate	22

LIST OF APPENDICES

APPENDIX 1	RESULTS	16
APPENDIX 2	EC-VALUES	22
APPENDIX 3	DETERMINATION OF NOELR	23
APPENDIX 4	CERTIFICATE OF ANALYSIS	24

1. STATEMENT OF GLP COMPLIANCE

Charles River Den Bosch BV, 's-Hertogenbosch, The Netherlands

All phases of this study performed by the test facility were conducted in compliance with:

- OECD Principles of Good Laboratory Practice;
- EC Council Directive 2004 (2004/10/EC, February 11, 2004, Official Journal of February 20, 2004).

The data generated and reported are considered to be valid.

Charles River Den Bosch

Signature:



Name: M.J.E. Desmares-Koopmans, Bachelor, ERT

Title: Study Director

Date: ab. December 2016..

2. TEST FACILITY QUALITY ASSURANCE STATEMENT

Charles River Den Bosch BV, 's-Hertogenbosch, The Netherlands.

Study title: Activated sludge respiration inhibition test (carbon and ammonium oxidation) with MLA-3202.

This report was inspected by the Charles River Den Bosch Quality Assurance Unit (QAU) according to the Standard Operating Procedure(s).

The reported method and procedures were found to describe those used and the report reflects the raw data.

During the on-site process inspections, procedures applicable to this type of study were inspected.

The dates of Quality Assurance inspections are given below.

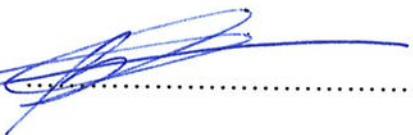
Project 511885

Type of Inspections	Phase/Process	Start Inspection date	End Inspection date	Reporting date
Study	Study Plan	31-May-2016	31-May-2016	31-May-2016
	Study Plan Amendment 01	20-Jul-2016	20-Jul-2016	20-Jul-2016
	Report	10-Oct-2016	10-Oct-2016	10-Oct-2016
Process	Environmental Science	19-Apr-2016	26-Apr-2016	26-Apr-2016
	Test Substance Handling			
	Exposure			
	Observations/Measurements			
	Test Substance Receipt	09-May-2016	20-May-2016	24-May-2016
	Test Substance Handling			

The review of the final report was completed on the date of signing this QA statement.

The facility inspection program is conducted in accordance with Standard Operating Procedure.

Charles River Den Bosch

Signature: 

Name: **Bart Kluskens, BSc**
Quality Assurance Auditor

Date: 6 December 2016

3. SUMMARY

The influence of MLA-3202 on the respiration rate of activated sludge was investigated after a contact time of 3 hours.

The study procedures described in this report were based on the OECD guideline No. 209, 2010. In addition, the procedures were designed to meet the test methods of the Council Regulation (EC) No. 440/2008 of 30 May 2008, Publication No. L142, Part C11 and ISO Standard 8192 (2007).

The batch of MLA-3202 tested, an UVCB, was a clear amber-red liquid. No correction was made for the purity/composition of the test item.

The test item was not sufficiently soluble to allow the preparation of a 10 g/L stock solution in water. Therefore, the test item and Milli-RO water mixtures were magnetically stirred for a period of approximately 24 hours. Subsequently, synthetic medium, sludge and Milli-RO water were added resulting in the required loading rates. Optimal contact between the test item and test medium was ensured applying continuous aeration and stirring during the 3-hour exposure period. Thereafter, oxygen consumption was recorded for approximately 10 minutes.

In a combined limit/range-finding test loading rates of 10, 100 and 1000 mg/L were tested. The highest loading rate was tested in triplicate, lower loading rates consisted of one replicate. Furthermore, at 1000 mg/L an abiotic control (1 replicate) and three replicates with a nitrification inhibitor were tested. Responses were compared to the blank and nitrification controls.

No statistically significant inhibition of the respiration rate of the sludge was recorded at a loading rate of 1000 mg MLA-3202 per litre.

The batch of activated sludge was tested for sensitivity with the reference item 3,5-dichlorophenol, and showed normal sensitivity.

The study met the acceptability criteria prescribed by the study plan and was considered valid.

MLA-3202 was not toxic to waste water (activated sludge) bacteria at a loading rate of 1000 mg/L (NOELR).

The ELR₅₀ was above 1000 mg/L.

4. INTRODUCTION

4.1. Study schedule

Experimental starting date : 09 June 2016
Experimental completion date : 10 June 2016

4.2. Purpose

The purpose of the study was to evaluate the test item for its ability to adversely affect aerobic microbial treatment plants, and if possible, to determine the ELR₅₀ and/or the no-observed effect loading rate (NOELR).

The endpoints of the study were based on total inhibition of the respiration (both heterotrophic and nitrification processes).

4.3. Guidelines

The study procedures described in this report are in compliance with the Organization for Economic Co-operation and Development (OECD), OECD guidelines for Testing of Chemicals, Section 2: Effects on biotic systems, Guideline no. 209, "Activated Sludge, Respiration Inhibition Test" (Carbon and Ammonium Oxidation), adopted 22 July 2010.

In addition, the procedures were designed to meet the test methods prescribed by the following guidelines:

- Council regulation (EC) No 440/2008 of 30 May 2008, Part C: Methods for the determination of ecotoxicity, Publication No. L142, C 11:"Biodegradation: Activated sludge respiration inhibition test", Amended by EC No. 2016/266 of 7 December 2015, Publication No. L54.
- ISO Standard 8192, Water Quality - Test for inhibition of oxygen consumption by activated sludge for carbonaceous and ammonium oxidation (2007).

4.4. Retention of records and materials

Records and material pertaining to the study, which include study plan and amendments, raw data, specimens, except perishable specimens, and the final report will be retained in the archives of the test facility for a minimum of 5 years after the finalization of the report. After this period, the sponsor will be contacted to determine how the records and materials should be handled. The test facility will retain information concerning decisions made.

Perishable specimens (e.g. requiring refrigeration or freezing) will be discarded following evaluation in the study without further notice to the study sponsor.

A sample of the test item will be retained until expiry date or applicable retest date. After this period the sample(s) will be destroyed.

4.5. Responsible personnel

4.5.1. Test facility

Study Director

M.J.E. Desmares-Koopmans, Bachelor, ERT

4.5.2. Sponsor Representative

Study Monitor

Audrey Batoon, Ph.D.

4.6. Definitions

Total respiration: Respiration by ammonium and carbon oxidation

Heterotrophic respiration: Respiration by carbon oxidation

Nitrification: Respiration by ammonium oxidation

Respiration rate: the oxygen consumption of aerobic activated sludge or waste water micro-organisms expressed generally as mg O₂ per litre per hour.

ATU: N-allylthiourea (nitrification inhibitor)

ECx/ELRx: the concentration/loading rate of the test item at which the respiration rate is x% of the respiration rate of the controls under the conditions of the test.

The **No Observed Effect Loading Rate (NOELR)** is the highest tested loading rate at which no effect is observed relative to the controls.

Activated sludge: the accumulated biological mass produced in the treatment of waste water by the growth of bacteria and other micro-organisms in the presence of dissolved oxygen.

Suspended solids (SS): the solids removed from activated sludge by filtration and dried to a constant mass, expressed in grams per litre.

5. MATERIALS AND METHODS

5.1. Test item

5.1.1. Test item information

Test item	207258/A
Identification	MLA-3202
Appearance	Clear amber-red liquid
Batch	RC-1045
Purity/Composition	UVCB
Test item storage	At room temperature
Stable under storage conditions until	17 February 2019 (expiry date)

For Certificate of Analysis see [APPENDIX 4](#).

5.1.2. Study specific test item information

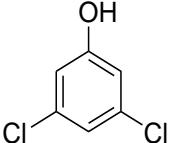
Purity/composition correction factor	No correction factor required
Test item handling	No specific handling conditions required
Stability at higher temperatures	Stable
Chemical name (IUPAC), synonym or trade name	Amides, tallow, N,N-bis(2-hydroxypropyl)
CAS Number	1454803-04-3
Specific gravity/density	0.9394
Solubility in water	< 1 g/L
Stability in water	Yes

5.2. Vehicle information

Not applicable

5.3. Reference item

5.3.1. Reference item information

Identification Number	RS92
Container	C1
Identification	3,5-Dichlorophenol
Structure	
Molecular formula	C ₆ H ₄ Cl ₂ O
Molecular weight	163.00
CAS Number	591-35-5
Description	Grey crystalline powder (determined at Charles River Den Bosch)
Batch	04631AJ
Purity	99.4%
Expiry date	09 September 2016
Storage conditions	At room temperature in the dark, desiccated
Stability under storage conditions	Stable
Supplier	Sigma-Aldrich Chemie GmbH, Steinheim, Germany

5.3.2. Reference item – preparation of stock and test concentrations

The batch of activated sludge was checked for sensitivity by testing the reference item 3,5-dichlorophenol.

A 3,5-dichlorophenol solution with a final concentration of 1 g/L in Milli-RO water was prepared. The pH as used for the test was 8.0. The 3,5-dichlorophenol stock solution was stored in a freezer until use. The reference item solution was defrosted at room temperature and diluted to reach the test concentrations. Three concentrations were tested: 2.0, 5.0 and 12 mg/L.

5.4. Test item – preparation of test solutions

The batch of MLA-3202 tested, an UVCB, was a clear amber-red liquid. No correction was made for the purity/composition of the test item.

The test item was not sufficiently soluble to allow the preparation of a 10 g/L stock solution in water. Therefore, 1-Litre test bottles were filled with 200 mL of test item mixtures in Milli-RO water (tap water purified by reverse osmosis; Millipore Corp., Bedford, Mass., USA) with initial loading rates of 2.5 times the final loading rate. These mixtures were stirred in closed dark brown bottles for approximately 24 hours. Subsequently, 16 mL synthetic medium made up to 50 mL with Milli-RO water and 250 mL sludge were added resulting in the required loading rates (see also paragraph 5.5). Optimal contact between the test item and test organisms was ensured applying continuous aeration and stirring.

5.5. Combined limit/range-finding test

In a combined limit/range-finding test loading rates of 10, 100 and 1000 mg/L were tested. The highest loading rate was tested in triplicate, lower loading rates consisted of one replicate. In addition, a blank control (6 replicates) and a nitrification control (2 replicates) were included. Furthermore, an abiotic control (1 replicate) and the highest loading rate with a nitrification inhibitor (3 replicates) were tested.

Table 1
Experimental set-up

Type of bottle	Test/reference item	Synthetic medium	Made up with Milli-RO water	Sludge
		Total volume mixture 250 mL		
Blank control (C)	no	16 mL	yes	250 mL
Nitrification control + ATU (CN)	no	16 mL	yes	250 mL
Reference item concentrations (R)	yes	16 mL	yes	250 mL
MLA-3202 loading rates (T)	yes	16 mL	yes	250 mL
MLA-3202 + ATU	yes	16 mL	yes	250 mL
Highest loading rate (TN)	yes	16 mL	yes	250 mL
Abiotic control of MLA-3202, Highest loading rate (TA)	yes	16 mL	yes	No sludge, made up to 500 mL with Milli-RO water

ATU: N-allylthiourea (Merck Schuchardt OHG, Hohenbrunn, Germany)

5.6. Test system

Test system	Micro-organisms in activated sludge.
Source	Municipal sewage treatment plant: 'Waterschap Aa en Maas', Heeswijk-Dinther, The Netherlands, receiving predominantly domestic sewage.
Preparation of the sludge	The sludge was coarsely sieved (1 mm) and allowed to settle. The supernatant was removed and ISO-medium was added. A small amount of the sludge was weighed and dried overnight at ca. 105°C to determine the amount of suspended solids (3.0 g/L of sludge, as used for the test). The pH was 7.1 on the day of testing. The batch of sludge was used one day after collection; therefore 50 mL of synthetic medium (=sewage feed) was added per litre of activated sludge at the end of the collection day. The sludge was kept aerated at test temperature until use.
Medium	Adjusted ISO-medium, formulated using RO-water (tap water purified by reverse osmosis; GEON Waterbehandeling, Berkel-Enschot, The Netherlands) with the following composition: CaCl ₂ .2H ₂ O 211.5 mg/L MgSO ₄ .7H ₂ O 88.8 mg/L NaHCO ₃ 46.7 mg/L KCl 4.2 mg/L
Rationale	Recognized by international guidelines as the recommended test system.

5.7. Test procedure and conditions

Contact time	3 hours, during which aeration and stirring took place.
Vessels	All glass open bottles/vessels.
Milli-RO water	Tap water purified by reverse osmosis (Millipore Corp., Bedford, Mass., USA).

Synthetic medium (=sewage feed)	16 g peptone 11 g meat extract 3 g urea 0.7 g NaCl 0.4 g CaCl ₂ .2H ₂ O 0.2 g MgSO ₄ .7H ₂ O 2.8 g K ₂ HPO ₄ Dissolved in Milli-RO water, made up to 1 litre and filtered. The pH was within 7.5 ± 0.5.
Inhibitor of nitrification	A 2.32 g/L solution of N-allylthiourea (ATU, Merck Schuchardt OHG) was prepared. 2.5 mL of this solution was added to 500 mL final test medium (final ATU concentration: 11.6 mg/L).
Air supply	Clean, oil-free air.
Aeration	The aeration was adjusted in such a way that the dissolved oxygen concentration at the start was above 60-70% saturation (60% of air saturation is > 5 mg/L at 20°C) and to maintain the sludge flocs in suspension.
Test set up	See paragraph 5.5 .
Performance of the test	The synthetic medium (16 mL) made up to 50 mL with Milli-RO and 200 mL test item solution were mixed (total volume 250 mL). The pH was determined. Thereafter 250 mL activated sludge was added. This was the start of the test.
	After the 3-hour contact time, the oxygen consumption was recorded for a period of approximately 10 minutes. During measurement, the sample was not aerated but continuously stirred on a magnetic stirrer.
	The pH was determined in the remaining part of the reaction mixture. This procedure was repeated for all test/reference item concentrations and controls.
	The medium temperature was recorded continuously in a temperature control vessel(s). The temperature control vessel(s) was/were identically prepared compared to the control vessels. A temperature control vessel with a REES sensor was placed in each fume cupboard of the climate room.
Oxygen recording	Determination of oxygen was performed with multiple oxygen probes connected to a BlueBox (GO-Systemelektronik GmbH, Germany), a multichannel measuring and controlling system.

5.8. Interpretation

5.8.1. Calculations

Calculation of oxygen uptakes

The respiration rate (R) from each vessel, in mg O₂/L.h was calculated or interpolated from the linear part of the respiration curve, which was generally between 2 and 7 mg O₂/L.

R was calculated by the BlueBox software as $(V_1 - V_2)/\Delta_t * 60$

Where:

V_1 =Value 1: the oxygen concentration at the start of the selected section of the linear phase (mg O₂/L),

V_2 =Value 2: the oxygen concentration at the end of the selected section of the linear phase (mg O₂/L),

Δ_t is the time interval between these two measurements.

Negative R values were expressed as 0 mg O₂/L.h ($V_1 < V_2$).

Furthermore the respiration rate was expressed as the amount of oxygen consumed per g dry weight of sludge per hour (R_s in mg O₂/g.h).

$$R_s = R / SS$$

Where SS is the concentration of suspended solids in the test mixture (g/L).

The different indices of R which may be combined are:

- S specific rate
- T total respiration rate
- N rate due to nitrification respiration (combined limit/ range-finding test)
- H rate due to heterotrophic respiration (combined limit/ range-finding test)
- A rate due to abiotic processes (combined limit/ range-finding test)
- B rate based on blank assays (mean)

Calculation of oxygen uptake due to nitrification

The relationship between total respiration (R_T), nitrification respiration (R_N) and heterotrophic respiration (R_H) is given below:

$$R_N = R_T - R_H$$

Where:

R_N is the rate of oxygen uptake due to nitrification (mg O₂/L.h).

R_T is the measured rate of oxygen uptake (no ATU) (mg O₂/L.h).

R_H is the measured rate of oxygen uptake with added ATU (mg O₂/L.h).

Calculation of the inhibition of the respiration rate

The percentage inhibition, I_T , of total oxygen consumption is given below:

$$I_T = [1 - (R_T/R_{TB})] \times 100\%$$

Similarly, the percentage heterotrophic oxygen uptake, I_H , is given below:

$$I_H = [1 - (R_H/R_{HB})] \times 100\%$$

Finally, the inhibition of oxygen uptake due to nitrification (if applicable), I_N , is given below:

$$I_N = [1 - (R_T - R_H) / (R_{TB} - R_{HB})] \times 100\%$$

Interpretation of results

Evaluation was based on the inhibition of the total respiration.

EC_x and ELR_x

For the reference item calculation of the EC₅₀ value was based on a 3-parameter normal CDF a non-linear regression analyses with the percentages of respiration inhibition versus the corresponding concentrations of the item.

For MLA-3202 no ELR₅₀-value could be calculated because the test item proved to be non-toxic (ELR₅₀ > 1000 mg/L)

NOELR determination

An effect was considered to be significant if statistical analysis of the data obtained for the test concentrations compared with those obtained in the blank control revealed significant inhibition of the respiration rate (Two-sample t-test, $\alpha=0.05$, one-sided, smaller).

No inhibition of the respiration rate was observed at any of the tested concentrations (for 1000 mg/L the mean value was considered). Therefore, the NOELR was considered to be the highest test concentration.

The calculations were performed with ToxRat Professional v. 3.2.1. (ToxRat Solutions® GmbH, Germany).

5.8.2. Acceptability of the test

1. The mean blank control oxygen uptake rate exceeded 20 mg oxygen per one gram of activated sludge (dry weight of suspended solids) in an hour (24 mg oxygen per one gram of activated sludge).

The coefficient of variation of oxygen uptake in blank control replicates did not exceed 30% at the end of the definitive test (4%).

2. The EC₅₀ of 3,5-dichlorophenol was in the accepted range of 2 to 25 mg/L for total respiration (6.1 mg/L, see also [APPENDIX 2](#)).

Since all criteria for acceptability of the test were met, this study was considered to be valid.

5.9. List of deviations

5.9.1. List of study plan deviations

1. The temperature was below the range prescribed by the study plan ($20 \pm 2^\circ\text{C}$) during the exposure phase, the temperature was 20.7-22.5°C.

Evaluation: The validity criteria for the controls and reference item were met and therefore this deviation is considered not to have influenced the sludge.

The study integrity was not adversely affected by the deviation.

5.9.2. List of standard operating procedures deviations

Any deviations from standard operating procedures were evaluated and filed in the study file. There were no deviations from standard operating procedures that affected the integrity of the study.

6. ELECTRONIC SYSTEMS FOR DATA ACQUISITION

The following electronic systems were used for data acquisition:

- REES Centron Environmental Monitoring system version SQL 2.0 (REES Scientific, Trenton, NJ, USA): Temperature.
- BlueBox Software version 3.4.2.0. (GO-Systemelektronik GmbH, Germany): Oxygen.

7. RESULTS

7.1. Inhibition of the respiration rate

Detailed study results are presented in [Table 3 of APPENDIX 1](#).

In the combined limit/range-finding test no statistically significant inhibition of the respiration rate of the sludge was recorded at a loading rate of 1000 mg MLA-3202 per litre. Thus the ELR₅₀ was above the highest loading rate tested (1000 mg/L).

There was no significant oxygen uptake from abiotic processes and the result at 1000 mg/L with a nitrification inhibitor showed that the heterotrophic inhibition of the respiration rate was not inhibited. Based on the results of the total and heterotrophic inhibition, nitrification was calculated to be 86% inhibited. Since, the heterotrophic respiration was stimulated and nitrification is calculated using the total and heterotrophic respiration rate the inhibition of nitrification may be overestimated. Since no distorted or biphasic dose-response curve for total respiration was observed, the effect on nitrification was not required according to the guidelines and was therefore not further investigated.

7.2. Determination of effect parameters

[Table 2](#) shows the effect parameters based on MLA-3202 loading rates.

Table 2
Effect parameters

Parameter	MLA-3202 Loading rate (mg/L)
NOELR	1000
ELR ₅₀	>1000

7.3. Experimental conditions

The pH values in the individual vessels are presented in [Table 3 of APPENDIX 1](#).

The pH in all test vessels, before addition of sludge was between 7.6 and 7.9. After the 3 hour exposure period the pH was between 7.5 and 8.0.

The temperature continuously measured in the temperature control vessels ranged between 20.7-22.5°C during the test, and was slightly outside the range prescribed by the study plan ($20 \pm 2^\circ\text{C}$, see also paragraph [5.9.1](#)).

8. CONCLUSION

Under the conditions of this present test MLA-3202 was not toxic to waste water bacteria (activated sludge) at a loading rate of 1000 mg/L (NOELR).

The ELR₅₀ was above 1000 mg/L.

APPENDIX 1**RESULTS****Table 3****Results: respiration rate/inhibition, pH values**

Replicate	Loading rate (mg/L)	pH		Respiration rate		% Inhibition respiration rate (mean value)
		Start	End	(mg O₂/L.h)	(mg O₂/g.h)¹	
C 1	0	7.9	7.7	36.40	24.27	
C 2	0	7.9	7.6	34.12	22.75	
C 3	0	7.6	7.6	37.24	24.83	
C 4	0	7.6	7.6	35.53	23.69	
C 5	0	7.6	7.6	33.68	22.45	
C 6	0	7.6	7.6	34.76	23.17	
C Mean				35.29 (R _{TB})	23.53	
SD				1.37	0.91	
CV (%)				4	4	
CN 1	0	7.9	8.0	16.29	10.86	
CN 2	0	7.6	7.9	22.22	14.81	
CN Mean				19.26	12.84	
R 1	2.0	7.9	7.9	22.20	14.80	37
R 2	5.0	7.9	7.8	21.26	14.17	40
R 3	12	7.9	7.8	12.57	8.38	64
T 1	10	7.8	7.5	50.35	33.57	-43
T 2	100	7.8	7.6	29.35	19.57	17
T 3a	1000	7.7	7.5	36.82	24.55	-4
T 3b	1000	7.7	7.5	39.01	26.01	-11
T 3c	1000	7.7	7.5	29.90	19.93	15
T3 Mean				35.24 (R _T)	23.50	0 (I _T)
TN a	1000	7.6	7.8	34.23	22.82	-78
TN b	1000	7.6	7.7	32.84	21.89	-71
TN c	1000	7.6	7.7	31.80	21.20	-65
TN Mean				32.96 (R _H)	21.97	-71 (I _H)
TA	1000	7.7	7.5	0 [#]	0 [#]	100

C: Blank control

¹ The amount of suspended solids in the final test mixture was 1.5 g/L.

CN: Nitrification control

R_{TB}: Total respiration blank

R: Reference item, 3,5-dichlorophenol

R_{HB}: Heterotrophic respiration in the nitrification control

T: Test item, MLA-3202

R_T: Total respiration with MLA-3202

TA: Abiotic control of MLA-3202

R_H: Heterotrophic respiration with MLA-3202

TN: MLA-3202 with N-allylthiourea

I_T: % inhibition of total respiration relative to R_{TB}

SD: Standard deviation

I_H: % inhibition of heterotrophic respiration relative to R_{HB}

CV: Coefficient of variation

*: Statistically significant compared to controls.

#: No respiration, therefore expressed as 0 mg O₂/L.h (see paragraph 5.8.1)

APPENDIX 1, continued
RESULTS

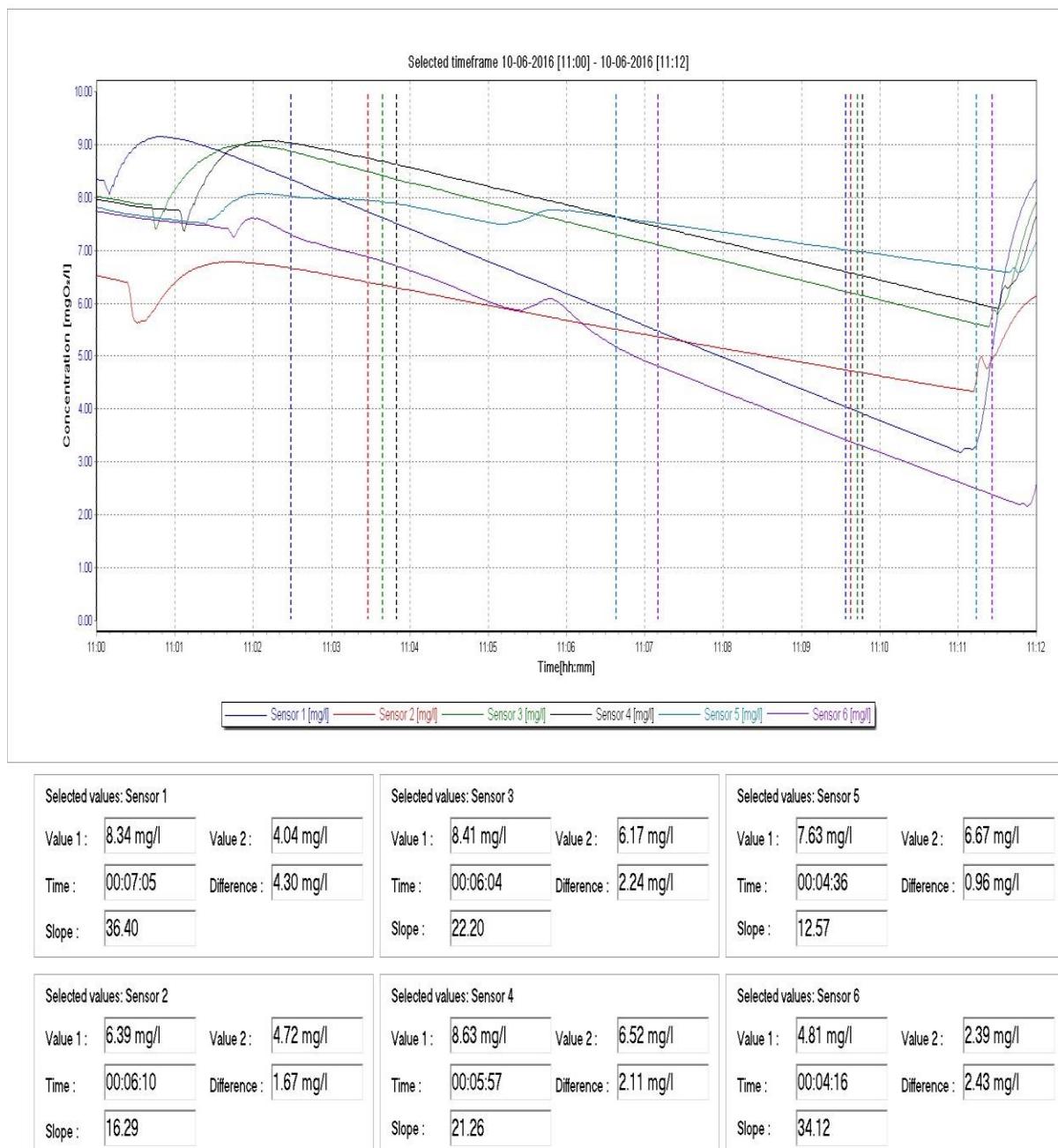
Relevant notes on Figure 1-4: Combined limit/range-finding test – Recording of oxygen consumption

The vertical lines represent the selected linear part of the respiration curve used for the calculation of the respiration rate.

Abbreviations used for the evaluation of the respiration curve:

- Value 1: Start of the linear part of the respiration curve selected for the calculation of the respiration rate (mg O₂/L).
- Value 2: End of the linear part of the respiration curve selected for the calculation of the respiration rate (mg O₂/L).
- Time: Time of the linear part of the respiration curve selected for the calculation of the respiration rate (h).
- Difference: Decrease of the oxygen concentration for the linear part of the respiration curve selected for the calculation of the respiration rate (mg O₂/L).
- Slope: Respiration rate (mg O₂/L h).

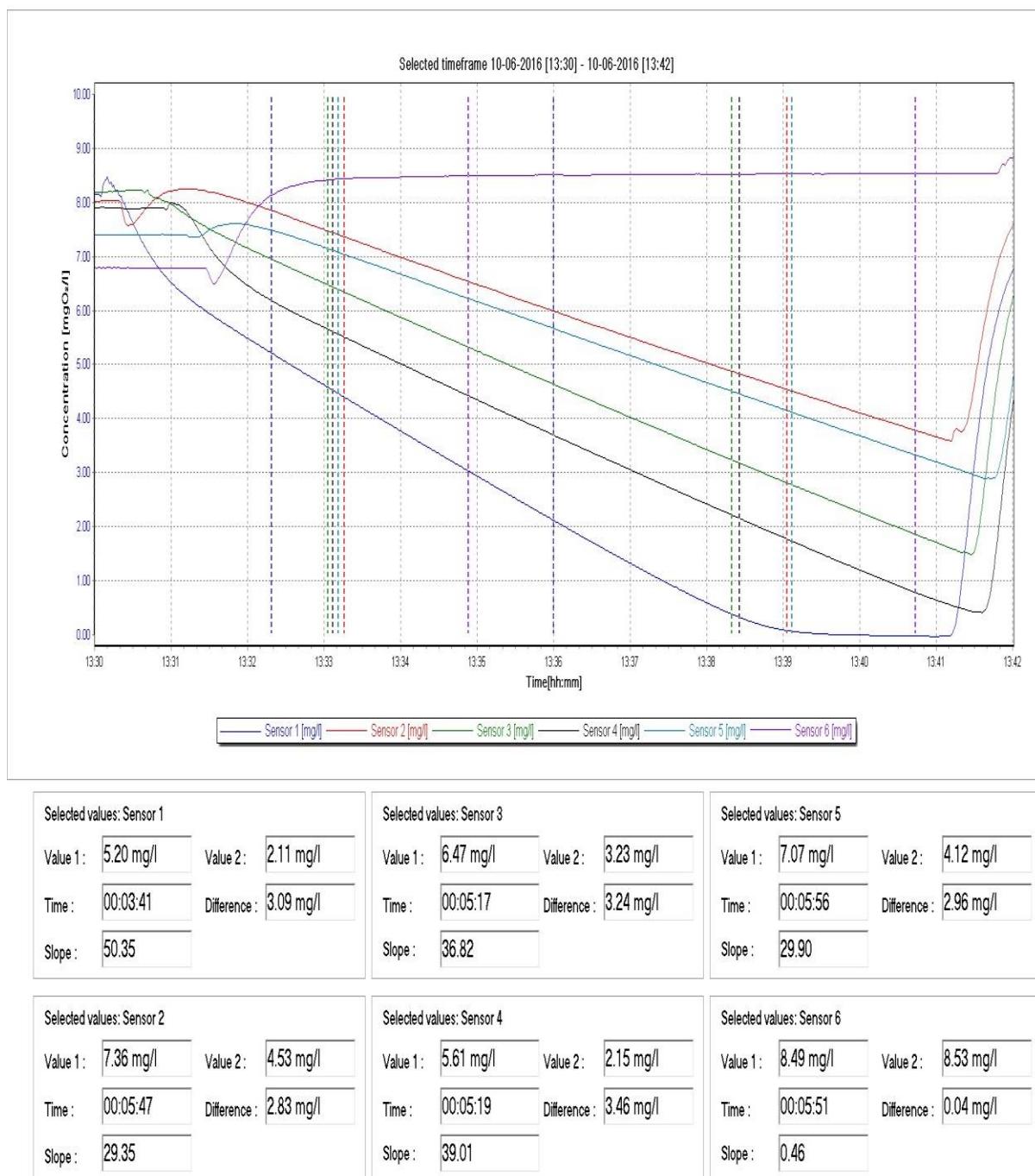
APPENDIX 1, continued
STUDY RESULTS



Probe	Code	Explanation Code
1	C 1	Blank control 1
2	CN 1	Nitrification control 1
3, 4, 5	R 1, R 2, R 3	Reference item 3,5-dichlorophenol (2.0, 5.0 and 12 mg/L)
6	C 2	Blank control 2

Figure 1
Recording of oxygen consumption (Blank/Nitrification controls, Reference item)

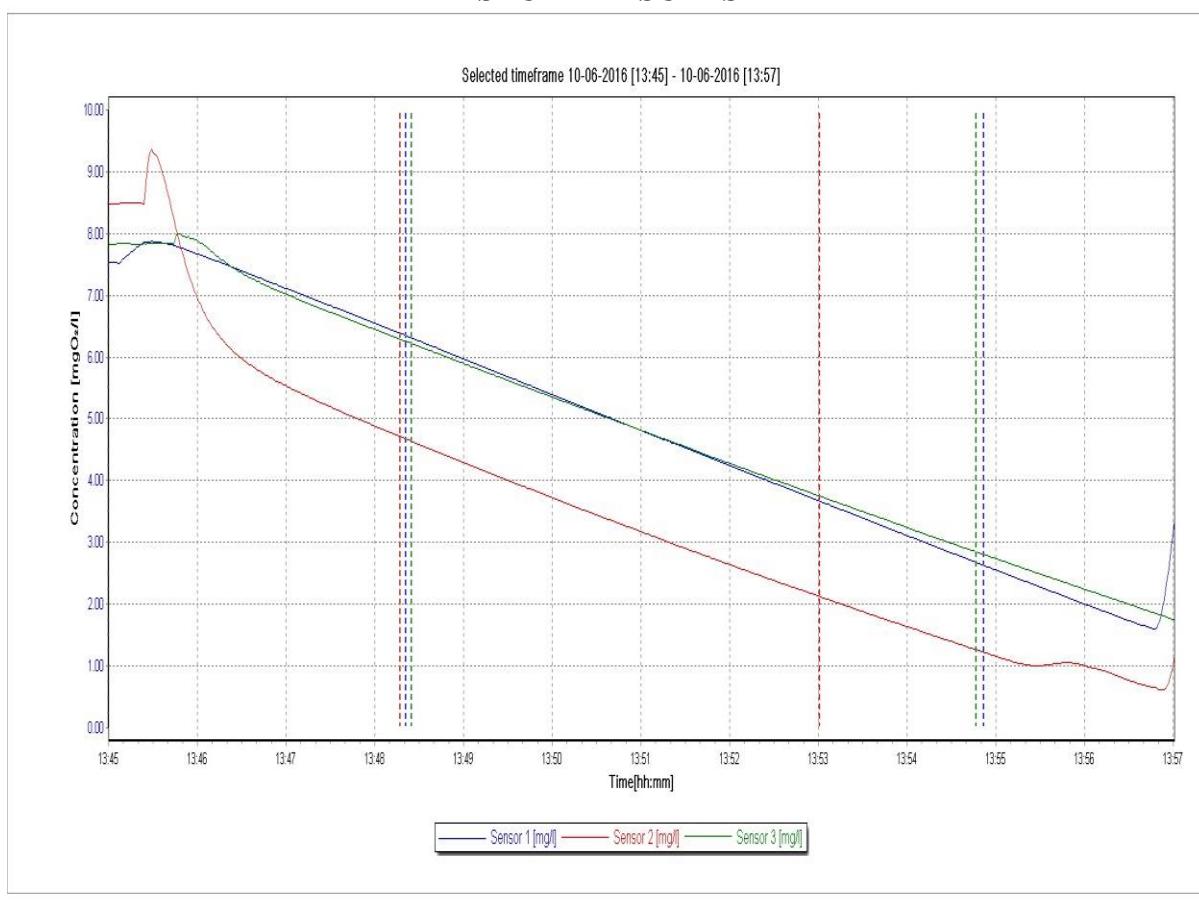
APPENDIX 1, continued
STUDY RESULTS



Probe	Code	Explanation Code
1	T 1	MLA-3202 (loading rate 10 mg/L)
2	T 2	MLA-3202 (loading rate 100 mg/L)
3, 4, 5	T 3a, T 3b, T 3c	MLA-3202 (loading rate 1000 mg/L)
6	T A	MLA-3202 (loading rate 1000 mg/L)

Figure 2
Recording of oxygen consumption (MLA-3202)

APPENDIX 1, continued
STUDY RESULTS

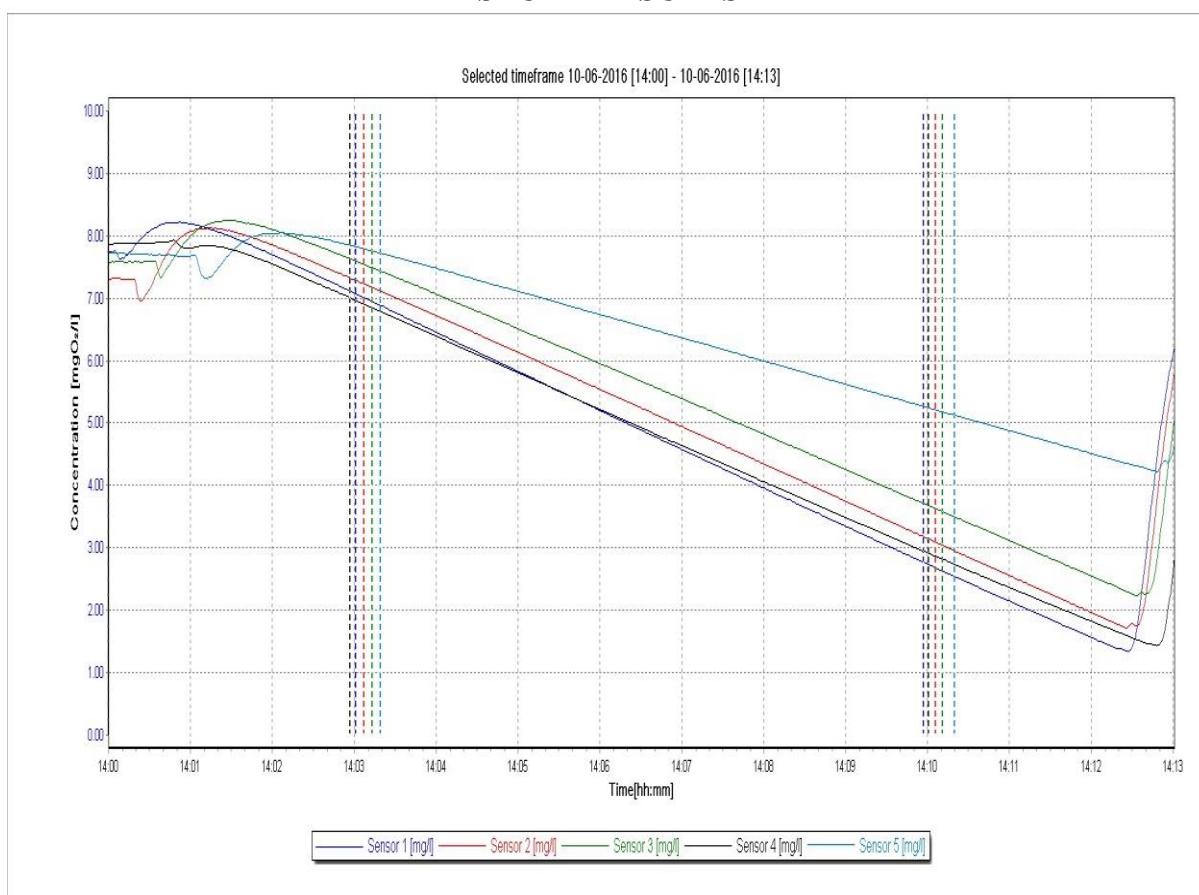


Selected values: Sensor 1	Selected values: Sensor 3	Selected values: Sensor 5
Value 1: 6.35 mg/l	Value 1: 6.22 mg/l	Value 1:
Value 2: 2.63 mg/l	Value 2: 2.85 mg/l	Value 2:
Time : 00:06:31	Time : 00:06:22	Time :
Difference: 3.72 mg/l	Difference: 3.37 mg/l	Difference:
Slope: 34.23	Slope: 31.80	Slope:
Selected values: Sensor 2	Selected values: Sensor 6	Selected values: Sensor 6
Value 1: 4.71 mg/l	Value 1:	Value 1:
Value 2: 2.12 mg/l	Value 2:	Value 2:
Time : 00:04:44	Time :	Time :
Difference: 2.59 mg/l	Difference:	Difference:
Slope: 32.84	Slope:	Slope:

Probe	Code	Explanation Code
1-3	T N a - c	MLA-3202 (loading rate 1000 mg/L) + ATU

Figure 3
Recording of oxygen consumption (MLA-3202 with nitrification inhibitor)

APPENDIX 1, continued
STUDY RESULTS



Selected values: Sensor 1	Selected values: Sensor 3	Selected values: Sensor 5
Value 1: 7.07 mg/l	Value 1: 7.49 mg/l	Value 1: 7.72 mg/l
Value 2: 2.77 mg/l	Value 2: 3.58 mg/l	Value 2: 5.12 mg/l
Time : 00:06:56	Time : 00:06:58	Time : 00:07:01
Difference : 4.30 mg/l	Difference : 3.91 mg/l	Difference : 2.60 mg/l
Slope : 37.24	Slope : 33.68	Slope : 22.22
Selected values: Sensor 2	Selected values: Sensor 4	Selected values: Sensor 6
Value 1: 7.23 mg/l	Value 1: 7.00 mg/l	Value 1:
Value 2: 3.09 mg/l	Value 2: 2.91 mg/l	Value 2:
Time : 00:06:59	Time : 00:07:04	Time :
Difference : 4.14 mg/l	Difference : 4.09 mg/l	Difference :
Slope : 35.53	Slope : 34.76	Slope :

Probe	Code	Explanation Code
1-4	C 3-6	Blank control 3, 4, 5, 6
5	CN 2	Nitrification control 2

Figure 4
Recording of oxygen consumption (Blank / Nitrification controls)

APPENDIX 2

EC-VALUES

Table 4
Estimated parameters of the 3-param. normal CDF

Std. Err.: standard error; 95%LCL|UCL: 95%-lower|upper confidence limits; t: t-statistic ($H_0: b_0|b_1|b_2 = 0$); p(t): probability that the deviation from zero is due to chance ($b_1 = \log EC_{50}$)

Parameter	Value	Std. Err.	95%LCL	95%UCL	t	p(t)
b0	23.523	0.496	22.310	24.736	47.461	<0.001
b1	0.783	0.093	0.555	1.010	8.411	<0.001
b2	1.108	0.308	0.354	1.863	3.596	0.006

Stop Reason = Converged (Optimization method: Levenberg-Marquardt)

R²: 0.961; adjusted R²: 0.949

Residual standard error: 1.20415

Akaike Criterion (AIC): 26.695

Shapiro Wilk's test on normal distribution of residuals: p = 0.346.

Table 5
Analysis of Variance for the 3-param. normal CDF

Source: source of variance; SS: sum of squares; df: degrees of freedom; MSS: mean sum of squares; F: test statistic; p(F): probability that the variance explained by the regression is due to chance (CDF: cumulative distribution function)

Source	SS	df	MSS	F	p(F)
Regression	263.92	2	131.96	91.007	<0.001
Residuals	8.70	6	1.45		
Total	274.49	8			

Since p(F|Regression) <= 0.05, a significant amount of variance is explained by the regression model.

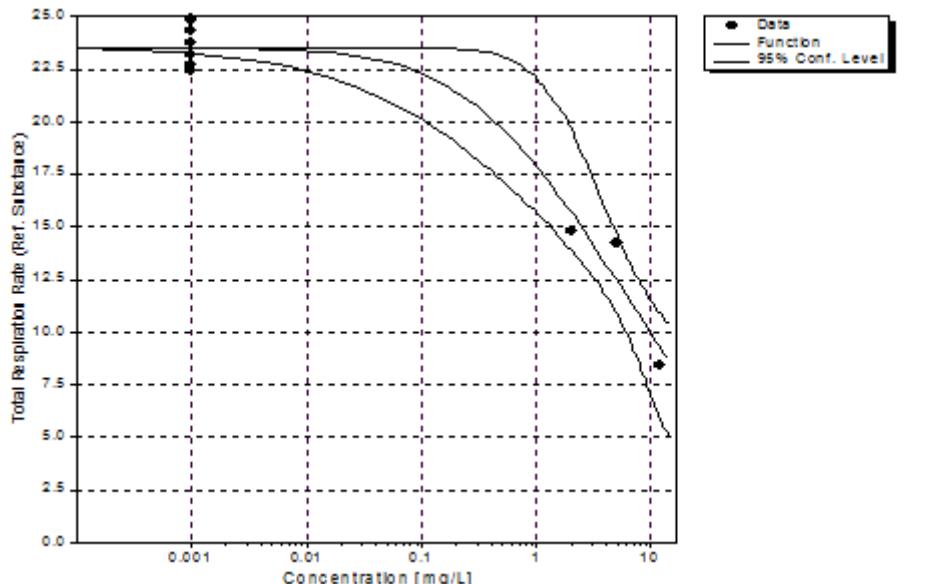


Figure 5
Concentration-effect curve showing the influence of 3,5-dichlorophenol on total respiration rate

Table 6
EC-values determined for 3,5-dichlorophenol

Parameter	EC ₅₀
Value [mg/L]	6.1
95%-confidence limits	3.6 - 10

APPENDIX 3

DETERMINATION OF NOELR

Test for normality of distribution

Shapiro-Wilk's Test on Normal Distribution

Mean: arithmetic mean; n: sample size; p(ShapiroWilk's W): probability of the W statistic (i.e. that the observed deviations from the normal distributions are due to chance). In case p(ShapiroWilk's W) is greater than the chosen significance level, the normality hypothesis(H₀) is accepted.

Treatm. [mg/L]	Mean	s	n
Control	23.526	0.9109	6
1000	23.496	3.1702	3

Results:

Number of residuals = 9

Shapiro-Wilk's W = 0.955

p(W) = 0.656

p(W) is greater than the selected significance level of 0.010; thus treatment data do not significantly deviate from normal distribution.

Normality check was passed (p > 0.01).

Test for homogeneity of variance

Levene's Test on Variance Homogeneity (with Residuals)

Source: source of variance; SS: sum of squares; df: degrees of freedom; MSS: mean sum of squares; F: test statistic; p: probability that the variance explained by the treatment is due to chance

Source	SS	df	MSS	F	p(F)
Treatment	5.38163	1	5.38163	9.204	0.019
Residuals	4.09273	7	0.58468		
Total	9.47440	8			

The Levene test indicates variance homogeneity (p > 0.010).

Variance homogeneity check was passed (p > 0.01).

Normal-distribution and variance-homogeneity requirements are fulfilled.

A parametric multiple test is advisable.

Determination of NOELR

Two-sample t-test Procedure

Mean: arithmetic mean; n: sample size; s: standard deviation; MDD: minimum detectable difference to Control (in percent of Control); t: sample t; p(t): probability of sample t for ; the differences are significant in case p(t) <= Alpha (The residual variance of an ANOVA was applied; df = N - k; N: sum of treatment replicates n(i); k: number of treatments).

Treatm. [mg/L]	Mean	s	df	%MDD	t	p(t)	Sign.
Control	23.526	1.8612					
1000	23.496	1.8612	7	-10.6	-0.02	0.491	-

-: non-significant

There is no statistically significant difference between Control and 1000 mg/L.

APPENDIX 4
CERTIFICATE OF ANALYSIS



Chemtura Corporation
12 Spencer St
Naugatuck, CT 06770

Analytical Services
www.chemtura.com

Certificate of Purity

Customer: Support for Toxicology Studies

Test Substance Name: MLA3202; Amides, tallow, N,N-bis(2-hydroxypropyl)

Physical Appearance: Liquid

CAS No.: 1454803-04-3

Ref. or Lot Number: RC-1045

Date of Analysis: revised March 18, 2016 (original issue March 7, 2016)

Percent Composition	Monoisotopic Mass (daltons)	Formula	Structure/ Identity
33.1	397.4	C ₂₄ H ₄₇ NO ₃	C18:1 (oleic) tallow amides, N,N-bis(2-hydroxypropyl)
22.9	371.3	C ₂₂ H ₄₅ NO ₃	C16:0 (palmitic) tallow amides, N,N-bis(2-hydroxypropyl)
13.6	395.4	C ₂₄ H ₄₅ NO ₃	C18:2 (linoleic) tallow amides, N,N-bis(2-hydroxypropyl)
11.0	399.4	C ₂₄ H ₄₉ NO ₃	C18:0 (stearic) tallow amides, N,N-bis(2-hydroxypropyl)
6.0	369.3	C ₂₂ H ₄₃ NO ₃	C16:1 (palmitoleic) tallow amides, N,N-bis(2-hydroxypropyl)
3.2	419.3	C ₂₆ H ₄₅ NO ₃	C20:4 (eicosatetraenoic) tallow amides, N,N-bis (2-hydroxypropyl)
2.0	393.3	C ₂₄ H ₄₃ NO ₃	C18:3 (linolenic) tallow amides, N,N-bis(2-hydroxypropyl)
1.5	282.3	C ₁₈ H ₃₄ O ₂	C18:1 (oleic) acid
1.1	421.4	C ₂₆ H ₄₇ NO ₃	C20:3 (eicosatrienoic) tallow amides, N,N-bis (2-hydroxypropyl)
5.6			Sum of residual components (< 1% each)
100.0			Total

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